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ULTRASTRUCTURAL, CYTOCHEMICAL AND MORPHOMETRIC  
ANALYSIS OF LIVER AND KIDNEY

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19. ABSTRACT (Continue on reverse if necessary and identify by block number) This investigation is designed to determine the ultrastructural effects of diethylnitrosamine (DENA) and trichloroethylene (TCE) on hepatic and renal tissues of the Japanese medaka, <i>Oryzias latipes</i> . Another objective of the study is to ascertain whether DENA can function as an inducer of cellular transformation for TCE. One group of 60 medaka was exposed to DENA (10mg/L) for 48hrs, rinsed and maintained in fresh water. Another group of fish was exposed continuously to TCE (10mg/L) until sacrificed at one of the scheduled time points. A third group was exposed to DENA (10mg/L) for 48hrs, rinsed in fresh water and subsequently exposed continuously to TCE (10mg/L) until the time of sacrifice. A control group of medaka was maintained in fresh water. The sacrifices were conducted at 8, 16, 32, and 52 weeks subsequent to the initial exposure to DENA. All tissues have been processed according to the appropriate procedures. Tissue samples from TCE/DENA exposed animals representing week 32 have been examined by transmission electron microscopy. Liver and kidney from control medaka have been examined and photographed, but will not be described in this abstract. While the					
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majority of parenchymal cells observed from fish exposed to TCE/DENA displayed no discernible effects of exposure, isolated hepatocytes and focal sites of cellular clusters were characterized by specific indicators of cytotoxicity. Livers from three fish were distinguished by the presence of multilocular cyst-like lesions that resemble spongiosis hepatitis. Peroxisomes of hepatocytes were successfully marked by the cytochemical procedures selected for the study. Several of the examined kidneys displayed features of pronounced cytotoxicity predominately in region II of the proximal tubule.

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In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

William D. Dotson  
PI Signature

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## INTRODUCTION

The primary methods currently established for analyzing the carcinogenicity of chemicals are tests on mammals such as mice and rats. However, such investigations require substantial laboratory space and research funds and involve prolonged periods of time. These features, in essence, preclude expanded efforts to search for carcinogenic compounds among the various substances present in the environment. Since there appears to be a strong correlation between mutagenicity and carcinogenicity, an effective and reproducible method of testing for mutations has been developed in bacteria (Ishidate, 1980). However, there are significant differences between bacteria and mammals with regard to biochemical pathways and cellular responses to xenobiotics. Appropriately, there is a need to develop an acceptable intermediate model, representative of a position between bacteria and rodents, for the analysis of potential chemical carcinogens in man.

Investigators representing a variety of disciplines currently employ fish models as routine and reliable bioassay testing systems for the detection of toxins and/or carcinogens in the environment, and more appropriately, as rapid indicators of carcinogenicity (Masahito et al., 1988; Harada et al., 1988; Klaunig et al., 1979). Several species of fish have exhibited relative degrees of susceptibility to a variety of carcinogens. The Japanese medaka, Oryzias latipes has served as a popular model for carcinogenicity studies, many of which have included diethylnitrosamine (DENA), a site-specific carcinogen for hepatic

neoplasms and methylazoxymethanol-acetate, a compound capable of inducing neoplasms in the liver, pancreas and kidney (Hinton et al., 1988; Harada et al., 1988; Norton & Gardner, 1987; Kyono, 1978). The acquisition of basic histological and ultrastructural information has provided some knowledge into the structural changes associated with neoplasms. However, the precise mechanism of transformation and the events associated with the development of pre-neoplastic lesions, including relative metabolic processes, uptake of molecules and DNA interactions are not completely understood.

This study is designed to determine the ultrastructural and selected cytochemical effects on the liver and kidney of an initial acute exposure of medaka to DENA followed by a chronic exposure of the fish to trichloroethylene (TCE). Another objective of the investigation is to analyze the effects of DENA and TCE, independently, on hepatic and renal tissue of the medaka subsequent to acute and chronic exposures, respectively. A critical phase of the study is to determine whether DENA can function as an initiator or inducer of carcinogenesis in combination with TCE. The phenomenon of tumor promotion illustrates the synergistic interaction of cancer-inducing agents. The two stage theory of cancer formation requires the initial exposure of tissue to a carcinogen, the initiator and the subsequent presence of a promoter, a compound which is not carcinogenic when acting independently.

Trichloroethylene was selected as a compound for study because of its potential as an environmental contaminant. The chemical is a constituent of numerous toxic waste sites and is

considered a serious risk to aquifers. Virtually no information is available on the synergism which may result from the combining of TCE and other toxic substances.

The three enzyme systems selected for examination, acid phosphatase, peroxidase and magnesium-dependent adenosine triphosphatase are detected by means of electron-dense visual markers. Pre-neoplastic lesions and hepatocellular carcinomas which develop subsequent to the administration of DENA have demonstrated altered activity of several enzymes, including adenosine triphosphatase (Hinton et al., 1988) and acid phosphatase (Fischer et al., 1983).

#### MATERIALS AND METHODS

Medaka cultures were maintained at the U.S. Army Biomedical Research and Development Laboratory, Fort Detrick, Frederick, Maryland. Sixteen day old medaka were divided into six major groups of approximately sixty fish each. One group of fish was exposed to DENA (10 mg/L) for 48 hours in a sealed container. This concentration was selected from range finding studies and is near the solubility limit for DENA. The fish were then rinsed and held in a 10 gallon tank with a flow rate of 100 ml/min. Another group was exposed continuously to TCE (10 mg/L). A third group was exposed to DENA (10 mg/L) for 48 hours in a sealed container, rinsed in fresh water and exposed continually



to TCE (10 mg/L) until sacrifice. The latter two groups of fish were maintained in 10 gallon tanks with a flow rate of 100 ml/min. Trichloroethylene was added to the tanks at a rate to ensure a constant concentration of 10 mg/L. The remaining groups were cultured in a similar fashion in 10 gallon tanks.

All fish were reared on a photoperiod regime of 16h light and 8h darkness. The feeding schedule included Tetramin twice daily, ocean plankton 4 times a week and brine shrimp 3 days a week. Nematodes were also a component of the diet with the amount given dependent upon the age of the fish.

At 8, 16, 32 and 52 weeks subsequent to the initial exposure to TCE, fifteen fish from each group were shipped to Southeastern Louisiana University and received within 24 hours. Within 48 hours of the final exposure to DENA the fish were anesthetized with tricaine methanesulfonate and the livers and kidneys excised. Ideally, ultrastructural investigations of the liver would dictate fixation by perfusion. However, the relatively small size of medaka, approximately 4 cm, has hindered the development of a reproducible and effective method of fixation by perfusion. Consequently, fixation was conducted by immersion. The tissues were processed according to the following protocols.

#### Transmission Electron Microscopy

Sections of tissue to be examined by conventional transmission electron microscopy were cut from each organ of interest, minced and placed in a cold solution of 0.1M sodium cacodylate (pH 7.4) buffered 4% glutaraldehyde. After 2 hours of fixation, the tissues were rinsed several times in fresh

buffer and post-fixed in cacodylate buffered 2% osmium tetroxide for 1 hour. The tissues were rinsed in buffer, dehydrated in a graded series of ethanol and embedded in POLY/BED 812. Thin-sections approximately 800 Angstroms thick were cut with an ultramicrotome and stained with lead citrate and uranyl acetate. Tissues have been examined and photographed with a JEOL 100S transmission electron microscope at 80kV.

### Scanning Electron Microscopy

Kidney and liver samples selected for analysis by scanning electron microscopy were fixed for 2 hours in a 0.1M sodium cacodylate buffered (pH 7.4) solution of 4% glutaraldehyde. Subsequent to several rinses in fresh buffer, the tissues were post-fixed for 2 hours in cacodylate buffered 2% osmium tetroxide. The tissues were rinsed and stored in buffer. Several of the samples have been selected for further processing. Those chosen were dehydrated in a graded series of ethanol, critical-point-dried with liquid carbon dioxide and coated with a thin layer of gold by an ion sputtering unit. The samples will be examined and photographed with an AMRAY 1200B scanning electron microscope at 30kV.

### Cytochemistry

All methods chosen for the cytochemical analysis of tissues have been described by Lewis and Knight (1986). Tissues selected for cytochemical analysis were cut into 50 micron thick sections by means of a vibratome. Controls for all cytochemical evaluations were represented by tissues placed in an incubation medium which lacked the appropriate substrate.

Peroxidase: Portions of liver were fixed for 1 hour in a cold 0.1M phosphate buffered (pH 7.2) solution of 2% glutaraldehyde. After several washes in fresh buffer, sections of tissue were cut by a vibratome and incubated for 1 hour at 37 degrees C in a solution comprised of 0.1M phosphate buffer (pH 7.2), 0.07mM 3,3'-diaminobenzidine and 1% hydrogen peroxide. The sections were washed in buffer and post-fixed for 1 hour in phosphate buffered (pH 7.2) 2% osmium tetroxide. The specimens were rinsed in buffer, dehydrated in ethanol and embedded in POLY/BED 812. Tissues from the longest exposure period (52 weeks) have been thin-sectioned and examined by transmission electron microscopy.

Acid phosphatase: Sections of liver were fixed for 1 hour in a cold 0.06M sodium cacodylate buffered (pH 7.2) solution of 2.5% glutaraldehyde. The specimens were rinsed several times in buffer and subsequently cut into sections by a vibratome. The tissues were incubated for 1 hour at 37 degrees C in a solution comprised of 1.25% sodium glycerophosphate, 2% magnesium chloride, 0.2M Tris/maleate buffer (pH 9.0), 1% lead nitrate and distilled water. After several rinses in buffer, the tissues were post-fixed for 45 minutes in a cacodylate buffered (pH 7.0) solution of 1% osmium tetroxide. The tissues were rinsed, dehydrated and embedded in POLY/BED 812. Thin-sections have not yet been cut and observed.

Adenosine triphosphate: Portions of liver and kidney were fixed for 1 hour in a cold 0.06M sodium cacodylate buffered (pH 7.0) solution of 2.5% glutaraldehyde. The fixed tissues were rinsed several times with fresh buffer and cut into sections with

a vibratome. The specimens were incubated for 1 hour at 37 degrees C in a solution consisting of 3mM ATP, 3mM magnesium sulfate, 3mM lead nitrate and 0.2M Tris/maleate (pH 7.4). The tissues were rinsed thoroughly in buffer and post-fixed for 1 hour in cacodylate buffered (pH 7.4) solution of 1% osmium tetroxide. The fixed tissues were rinsed in buffer, dehydrated and embedded in POLY/BED 812.

## RESULTS

All scheduled sacrifices have been conducted and the tissues processed according to the appropriate protocols. A significant number of fish from each of the tanks that contained exposed organisms died prior to the final sacrifice. In fact, only 2 fish that experienced an acute exposure to DENA survived for the 52 week period, while 3 fish in each of the TCE and DENA/TCE tanks remained alive for the duration of the study. Fish sacrificed at the 52 week period will be subjected only to a morphological evaluation since so few specimens survived to the final sacrifice. At least 9 fish in each of the control tanks survived until the designated time of sacrifice.

Chemical fixation of all tissue has been adequate, with little evidence of fixation related artifacts. Samples of kidney and liver from both control and tissues exposed to TCE and DENA alone that were excised from fish sacrificed at week 32 of

the investigation have been examined by transmission electron microscopy and will be reported in this document. The liver of control medaka has been described in a previous annual report; therefore, only the basic ultrastructural features will be reiterated in this report.

#### Control Tissue

The liver of control medaka was comprised of hepatocytes aligned in a tubulosinusoidal pattern. Plates 2 cells thick extended out from a central vein. The space of Disse, attenuated endothelial cells and the sinusoidal lumen were positioned at the basal region of hepatocytes. Bile preductular cells formed significant segments of intralobular biliary ductules.

Large, centric nuclei of hepatocytes contained sparse quantities of heterochromatin that tended to associate with the nuclear envelope. Nucleoli were distinguished by clusters of granular elements separated by anastomosing nucleolonemas. Lipid droplets were dispersed at random, while lysosomes and peroxisomes were prevalent. Cylindrical mitochondria were characterized by short, narrow cristae and an electron-opaque matrix. The rough endoplasmic reticulum was arranged as flattened cisternae of variable length with no detectable pattern of organization.

Bile preductular cells were long and narrow in appearance and displayed oval nuclei, many of which were indented. Substantial quantities of both euchromatin and heterochromatin were dispersed in the nucleus. Tonofilaments were extensive in number and expanded in various planes. Desmosomes were a

frequent feature between adjacent hepatocytes and bile preductular cells.

The kidney of medaka was comprised of proximal convoluted tubules that could be divided, structurally, into two distinct regions. Region I was distinguished by the presence of prismatic cells with large, slightly oblong nuclei. A prominent brush border was evident and pronounced secondary lysosomes vacuoles were concentrated apically. Basal and lateral enfolds of the plasma membrane were features of the cells, while junctional complexes were present at the apical zone.

Epithelial cells of region II contained spherical nuclei which were positioned distally. The proximal zone possessed numerous electron-dense granules of various diameters, while substantial numbers of small vesicles concentrated distally. Free ribosomes were dispersed throughout the cytoplasm and small, cylindrical mitochondria tended to localize in the proximal zone. The brush border appeared to be shorter in length than that of region I. No secondary lysosomes were detected. Junctional complexes were formed at cellular borders.

#### Tissues Exposed to DENA/TCE

Medaka selected for histological preparation have not yet been processed; therefore, this document will report only the ultrastructural manifestations of exposure. In addition, since a morphometric analysis of the tissues has not yet been completed, the results will concentrate only on an evaluation of the structural and cytochemical changes that have taken place in hepatic and renal tissues. Medaka sacrificed at week 32 will be

described in this report.

The majority of parenchymal cells displayed no discernible effects of exposure. However, isolated hepatocytes and focal zones consisting of cellular clusters were characterized by specific indicators of cytotoxicity. The most pronounced nuclear alteration concerned a dissociation of the outer nucleolonema and a concomitant dispersion of granular elements. Structural changes of rough endoplasmic reticulum included moderate to extensive dilation of cisternae. The swollen cisternae were characterized by a reduction in the number of attached ribosomes.

Although the morphometric analysis of cellular organelles has not been completed, general observations indicate there have been no significant changes of the peroxisome or lysosome content of the affected parenchymal cells. Control and exposed hepatocytes frequently displayed cleft-like zones. Swollen mitochondria with vesiculated cristae were evident in many of the cells. Mitochondria were also observed with myeloid figures associated with the outer membrane.

Several of the livers displayed distinct regions that consisted of small to medium sized cells of an uncharacterized nature. The pleomorphic nuclei usually expressed a high euchromatin/heterochromatin ratio. The cells possessed relatively few cisternae of rough endoplasmic reticulum and substantial quantities of Golgi complexes, vesicles and lysosomes. Large amounts of collagen fibers were dispersed among the intercellular spaces. Peroxisomes, as expressed by cytochemical markers, were not a consistent cytoplasmic

feature of the cells comprising these regions. Bile preductular cells were not detected in the cellular clusters described above. However, such cells appeared to be structurally normal in areas of the liver not visually affected by DENA.

Three of the livers thus far examined displayed several focal sites of various dimensions that were characterized by multilocular cyst-like lesions termed spongiosis hepatitis. The structural framework of the spongiotic lesions was formed by attenuated cells with small irregular nuclei. The plasma membrane was extended to generate cytoplasmic processes. Concentrated arrays of randomly positioned intermediate filaments were prominent cytoplasmic features. The processes of adjoining cells were maintained in position by desmosomes. The cystic cavities contained fibrous or flocculent material.

There was no observable evidence of neoplasia in the kidneys of medaka exposed to DENA/TCE. Cytotoxicity was evident along the length of the proximal tubule. However, the more pronounced indicators of cellular necrosis and alteration were localized predominately in region II. The majority of proximal tubules examined appeared markedly distended as a result of the accumulation in the lumen of cellular debris, including myeloid figures, membrane fragments and isolated organelles.

Large apical blebs protruded from cells into the tubular lumen at focal sites along the length of region II. Focal sites of the apical plasma membrane appeared fragmented. Many of the cells displayed pyknotic nuclei.



## CONCLUSIONS

All phases of the project concerned with exposure of medaka to DENA and TCE have been completed. The scheduled sacrifices and subsequent fixations occurred without difficulty. However, since a significant number of fish from each experimental tank died just prior to the last sacrifice, several modifications may be implemented with regard to that phase of the investigation devoted to morphometry. A final decision will be made after the analysis of all remaining tissue.

The remainder of the investigation has proceeded without difficulty. Livers and kidneys from medaka sacrificed at week 32 have been analyzed by transmission electron microscopy for pertinent ultrastructural and cytochemical features. Tissue samples are currently under investigation by scanning electron microscopy.

Examinations of hepatic parenchymal cells for the presence of peroxisomes indicate the successful nature of the protocol followed for the detection of such organelles by means of a cytochemical marker. A morphometric analysis of the peroxisomes should be conducted without difficulty since the organelle is readily recognizable. The success of the cytochemical procedure was important since peroxisomes are frequently mistaken for lysosomes in the absence of a definable cytochemical marker. Consequently, any numerical analysis of either organelle constructed strictly on ultrastructural observations may be misleading and erroneous.

The detection of spongiosis hepatitis among livers exposed to

DENA is to be expected since it develops primarily in response to hepatocarcinogens. Initial ultrastructural examinations indicate the spongiotic lesions are organized in a manner that is consistent with structural descriptions in the literature. Attempts will be made to localize a spongiotic lesion for analysis by scanning electron microscopy to ensure a 3 dimensional profile of the DENA induced abnormality.

Although the initiation of the project was delayed approximately 6 months because of difficulties associated with rearing the medaka, the primary objectives of the investigation should be accomplished within the next year.

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